NONGENOMIC REGULATION OF PROTEIN KINASE C ISOFORMS BY VITAMIN D METABOLITES IN CHONDROCYTE MATRIX VESICLES AND PLASMA MEMBRANES

A
THESIS

Presented to the Faculty of
The University of Texas Graduate School of Biomedical Sciences
at San Antonio
in Partial Fulfillment
of the Requirements
for the Degree of

MASTER OF SCIENCE

By
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San Antonio, Texas

May 1996

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	9 Jan 97	

4. TITLE AND SUBTITLE
Nongenomic Regulation of Protein Kinase C Isoforms By Vitamin D Metabolites In Chondrocyte Matrix Vesicles And Plasma Membranes

5. FUNDING NUMBERS

6. AUTHOR(S)
Earl Bryan Ellis

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas

8. PERFORMING ORGANIZATION REPORT NUMBER
96-138

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
DEPARTMENT OF THE AIR FORCE
AFIT/CIA
2950 P STREET
WPAFB OH 45433-7765

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS

15. NUMBER OF PAGES
50

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

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This thesis would not have been possible without the enduring love, patience and encouragement of my wife Barbara. It was her sacrifice and devotion that saw me through this project. During the past three years of a very demanding residency I have been continually sustained by Barb's efforts and I will always be grateful to her. To my beautiful daughters Micah and Haley; I hope that the perseverance and dedication embodied in this thesis may someday encourage your pursuit of worthwhile ambitions. To my loving parents, Cecil and Nancy Ellis; your unfaltering confidence and support in all my endeavors have been my inspiration.

ACKNOWLEDGEMENTS

I am profoundly grateful for the guidance of Drs. Barbara Boyan and Zvi Schwartz who made this research endeavor possible. Their boundless energy, keen insight, and organizational skills are simply amazing. My daily association with Dr. Victor Sylvia was truly a pleasure. I found his appreciation and love of science to be both refreshing and contagious. I would also like to thank Ruben Gomez, Monica Luna and Hui Li for their laboratory expertise which was instrumental in the successful completion of this project. Lastly, a heartfelt thanks to all of the Willford Hall Department of Periodontics staff for the time and effort they have invested in my education.

NONGENOMIC REGULATION OF PROTEIN KINASE C ISOFORMS BY VITAMIN D METABOLITES IN CHONDROCYTE MATRIX VESICLES AND PLASMA MEMBRANES

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Normal biologic function of an organism requires communication and regulation at several levels. Perhaps the most fundamental communication is that which occurs between the extracellular matrix and the intracellular environment. Elucidation of the signal transduction pathways in the areas of chondrocyte, osteoblast and inflammatory regulation would have significant implications in wound healing, regenerative procedures, implants and treatment of metabolic diseases of bone and cartilage. The rat costochondral chondrocyte model has been used to demonstrate that vitamin D_3 metabolites can directly regulate many cellular and extracellular matrix functions and that this regulation is dependent both on the type of vitamin D metabolite, and on the maturation level of the chondrocytes. Matrix vesicles, distinct extracellular organelles critically involved in matrix mineralization, are also differentially regulated by vitamin D metabolites. Recently, it was shown that vitamin D metabolites can regulate protein kinase C activity in chondrocyte culture lysates and that these effects are also metabolite- and maturation-

specific. Protein kinase C (PKC) is a family of isozymes that are known to be important in cell signal transduction. The aim of this study was to examine whether PKC mediates the nongenomic effect of vitamin D on chondrocyte regulation. The present study investigated whether PKC activity was present in matrix vesicles (MV) and plasma membranes (PM) and whether this activity can be directly regulated by vitamin D metabolites. Ribcages from Sprague-Dawley rats were dissected to obtain resting zone (RC) and adjacent growth zone (GC) chondrocytes. Cells representing chondrocytes at two different states of maturation were cultured separately through four cell passages. Chondrocyte plasma membranes were obtained by differential centrifugation of cell homogenates. Matrix vesicles were isolated by differential centrifugation of a trypsin digest supernatant. Matrix vesicles contain no DNA or RNA; therefore, any effect of hormones directly on the MV will not involve new gene transcription or translation. PKC activity in samples was measured using a filter-binding assay and protein content was determined using the BCA method. Presence of PKC isoforms was determined in two ways. 1) Immunoprecipitation using isoform-specific-PKC antibodies, SDS gel electrophoresis and visualization via enhanced chemiluminescence were used to determine presence of specific PKC isoforms in MV and PM samples. 2) The presence of PKC in MV was further verified by immunogold labeling and transmission electron microscopy. Direct effect of vitamin D_3 metabolites was tested by incubation of isolated PM and MV with vitamin D metabolites or untreated controls for 9, 90, or 270 minutes. We found that PKC activity was present in MV and could be regulated by a PKC activator (bryostatin) and a PKC inhibitor (psuedosubstrate inhibitor peptide). Using specific antibodies for different PKC isoforms, it was determined that PKCα is the major isoform in chondrocyte culture lysates and isolated PMs and that PKCζ is present in MVs. Further verification of the existence of PKCζ in MV was accomplished using immunogold labeling and visualization at the TEM level. Isolated MV and PM incubated with 10^{-9}-10^{-8} M vitamin D_3 metabolites showed dose-dependent effects on PKC activity. In membranes isolated from GCs, 1,25-$(OH)_2D_3$ inhibited PKC activity in MV and stimulated PKC activity in PM. In membranes isolated from RCs 24,25-$(OH)_2D_3$ also inhibited PKC activity in MV and stimulated it in PM.

PKC specific activity was lipid-dependent, whereas enzyme activity in MV was not. The results indicate that PKC isoforms-α and -ζ are differentially distributed between MV and PM and are regulated by vitamin D metabolites in a membrane-specific manner. This study suggests that the nongenomic effect of vitamin D_3 on PM and MV is mediated by PKC and indicates a possible mechanism for the differential regulation of PM and MV enzymes observed in intact cultures. PKCζ may be important in nongenomic, autocrine signal transduction at sites distant to the cell.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

In biological terms, the development of life can be described as a complex interplay of proliferation and differentation events that proceed in a highly ordered manner. As a prerequisite for those events, cells must respond to extracellular signals with a specific set of mechanisms that regulate or modulate gene expression. Between the signal and the gene, a system of cellular components is assembled to guarantee a specific and successful process of signal transduction. Pathways of signal transduction, though differing remarkably in their complexity and in the use of cellular components, seem to obey certain principles which are evolutionarily conserved and widely distributed among living organisms. Clearly, a better understanding of the intricacies of biological signal transduction pathways is a desirable goal as evidenced by the shear volume of research being conducted in this area. This thesis addresses signal transduction in cartilage cells in response to vitamin D metabolites. Understanding the regulatory processes at work in these cells could have a profound impact on such processes as hard tissue wound healing, fracture repair, periodontal regeneration, endosseous implants and possibly even the development of new drugs to treat developmental disturbances of cartilage or bone.

A. Signal Transduction

A fundamental biological requirement for living organisms, tissues, and cells is that they are able to respond to stimuli. Individual cells must respond to extracellular signals in an appropriate manner so that proliferation and differentiation events can occur normally. The study of the complex mechanisms by which cells respond to extracellular signals and the resultant changes in intracellular events is the study of signal transduction.

One of the integral concepts in signal transduction is that of the of the "second messenger". The second messenger concept arose from landmark work by Sutherland who, in the mid-1960's, was studying catecholamine interactions with the plasma membranes of erythrocytes. He discovered that this interaction led to activation of adenylate cyclase (Stryer, 1981). The second messenger concept provided that an extracellular mediator or "first messenger" could bind to a receptor at the cell membrane which then led to stimulation of an intracellular "second messenger" (e.g. cyclic AMP). The "second messenger" in turn goes on to alter other processes within the cell. This concept has been refined and modified over the years but still remains valid today.

The current concepts of signal transduction were nicely outlined in a review paper by Hug and Sarre (1993). Extracellular signals, in the form of extracellular proteins, hormones, or growth factors, either penetrate the cellular membrane or bind to the extracellular domain of receptors. Activated receptors are capable of activating effectors, either directly or by means of changing the amount or intracellular distribution of so-called second messengers. These second messengers activate target proteins which, as such, or by action on further "downstream" targets, finally modulate gene expression at both the transcriptional and translational levels.

Most of the components of signal transduction pathways are proteins whose activity is altered by either ligand or second messenger binding, by covalent modifications and by subsequent changes in conformation or subunit number. The majority of covalent modifications observed are phosphorylations of various proteins. The group of enzymes which catalyze the addition of phosphate groups are called kinases.

In general, the important signal transduction enzymes are divided into serine/threonine kinases and tyrosine kinases on the basis of the phosphate-accepting residue in the substrate

protein (Hanks, *et al.*1988). Two prominent Ser/Thr-specific kinases, both activated by second messenger action, play a central role in signal transduction: the cyclic AMP-dependent protein kinase A (PKA) and the Ca^{+2}/phospholipid activated protein kinase C (PKC).

B. Protein Kinase C

Protein kinase C was the enzyme of interest for this thesis. Protein kinase C was first identified in rat brain extracts by Inoue and Nishizuka in 1977. It was reported as a calcium and phospholipid activated protein kinase and was eventually linked to signal transduction because diacylglycerol, one of the early products of inositol phospholipid breakdown, greatly increased the affinity of PKC for Ca^{+2} and its activity (Takai *et al.*, 1979).

Evidence that activation of PKC was linked to signal transduction has primarily come from experiments with platelets (Kawahara *et al.*, 1980). This work indicated that platelets, when stimulated, produce diacyglcerol (DAG) containing arachidonic acid, and that this reaction is accompanied by the disappearance of inositol phospholipids. The appearance of DAG in membranes was found to always be associated with the activation of PKC.

Several phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), are potent tumor promoters. Studies with many cell types suggest that phorbol esters act on the cell membrane surface (Blumberg, 1980). Castagna *et al.*, (1982) presented evidence that PKC is a target for phorbol esters since these agents directly activate PKC. Like diacylglyerol, TPA increases the affinity of PKC for Ca^{2+}, resulting in enzyme activation (Bell and Burns, 1991).

PKC activation is thought to be involved in a variety of important cell functions, including proliferation, gene expression, membrane transport, and excretion of hormones and neurotransmitters (Nishizuka, 1984). Currently 12 isoforms of PKC have been identified and are identified by Greek letters (α, βI, βII, γ, δ, ε, ζ, η,θ , τ, λ and μ) (Dekker and Parker, 1994).

While these isoforms constitute a family, studies show that there are functional differences between the members. In addition to different activation requirements, these isoforms are not uniformly distributed within tissues and have been shown to favor particular substrates to phosphorylate (Dekker and Parker, 1994). These isoforms would not be expected to function interchangeably, but to act in parallel to transduce specific signals to the cell (Parker *et al.*, 1989). PKC has recently been implicated in signal transduction processes mediated by vitamin D metabolites in chondrocytes. (Sylvia *et al.*, 1993). This thesis examined which protein kinase C isoforms are involved in these processes and also determined their relative distribution in matrix vesicles and plasma membrane fractions derived from chondrocytes.

C. Vitamin D

Vitamin D has been shown to be essential for proper endochondral ossification (Raisz and Kream, 1983). Vitamin D, or cholecalciferol, is made in the skin from an inactive form, 7-dehydrocholesterol, by reactions initiated by sunlight. Vitamin D is hydroxylated first in the liver and then in the kidney to an active form, 1,25 dihydroxycholecalciferol, which is important for calcium and phosphate metabolism (Stryer, 1981). In vitamin D-deficient chicks, the growth zone of the cartilage fails to mineralize. When given vitamin D (Atkin *et al.*, 1985), or when given calcium (Balsan *et al.*, 1986) the growth plate rapidly heals. Undeniably vitamin D metabolites are required for endochondral bone formation; however the mechanisms of how this hormone acts to communicate its signals to mineralizing cells their extracellular matrix are not yet understood.

D. Endochondral Ossification and vitamin D metabolites.

Endochondral bone formation involves the differentiation of mesenchymal stem cells into chondrocytes and subsequent mineralization of the cartilaginous matrix. Calcification of cartilage

is critical for long bone growth, fracture repair and bone induction and all these processes involve endochondral ossification (Glowacki, 1982; Ksiazek and Moskalewski, 1983).

During long bone development the cartilaginous epiphysis is separated from the diaphysis by the epiphyseal growth plate. Chondrocytes in the growth plate divide, mature, increase in size, and calcify their extracellular matrix (Boskey, 1981; Anderson, 1969). Rat chondrocyte cultures have been used to understand the differentiation and regulation of cartilage cells during endochondral ossification (Boyan *et al.*, 1992a). These cultures have proven to be an excellent model for studying the genomic and nongenomic effects of vitamin D metabolites, such as 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, on resting zone and growth zone chondrocytes (Boyan *et al.*, 1988a; Langston *et al.*, 1990; Schwartz *et al.*, 1988a, 1988b, 1992b; Swain *et al.*, 1992). This culture model has also been used to study the regulation of important extracellular organelles called matrix vesicles.

E. Matrix vesicles

Matrix vesicles are extracellular membrane bound organelles produced by chondrocytes, osteoblasts, odontoblasts and calcifying neoplasias in vivo (Anderson, 1969) and in culture (Boyan *et al.*, 1988b, 1990; Ecarot-Charrier *et al.*, 1988; Bonewald *et al.*, 1990; Slavkin *et al.*, 1992). These organelles contain a variety of enzymes. Most notably, matrix vesicles are enriched in alkaline phosphatase activity with respect to plasma membranes, and this characteristic has been used as a marker for matrix vesicles. Other plasma membrane enzymes are present in matrix vesicles, but their relative distribution is distinct from that of the cell membrane (Boyan *et al,.* 1988a). Matrix vesicle phospholipid metabolism is known to be regulated independently from that of the cell (Schwartz *et al.*, 1988a and b). Matrix vesicles also contain metalloproteinases capable of remodeling the extracellular matrix (Dean *et al.*, 1992,1994; Hirschman *et al.*,1983).

F. Regulation of Matrix Vesicles by Vitamin D

Production and enzyme activity of matrix vesicles is regulated by extracellular proteins, hormones and growth factors (Boyan *et al.*, 1992; Yang *et al.*, 1991; Schwartz *et al.*, 1992a and b; Nasatzky *et al.*, 1994). The rat chondrocyte model developed by Boyan and coworkers (Boyan *et al.*,1988) has also been used to look at mechanisms regulating matrix vesicle production and activity. These studies have shown that the response of chondrocytes to vitamin D metabolites is dependent on the state of cell maturation. That is, the more mature growth zone cells and less mature resting zone cells respond differently to vitamin D metabolites. The vitamin D metabolite, 1,25-$(OH)_2D_3$ stimulates alkaline phosphatase and phospholipase A_2 specific activity in matrix vesicles produced by growth zone chondrocytes, whereas the vitamin D metabolite, 24,25-$(OH)_2D_3$ increases alkaline phosphatase and inhibits phospholipase A_2 activity in matrix vesicles produced by resting zone chondrocytes (Boyan *et al.*, 1988,1989; Schwartz *et al.*, 1988,1990). Other aspects of chondrocyte metabolism are also differentially regulated by the vitamin D metabolites, including arachidonic acid turnover (Schwartz *et al.*, 1990; Swain *et al.*, 1992), Ca^{+2} ion flux (Langston *et al.*, 1990), prostaglandin production (Schwartz *et al.*, 1992), and protein kinase C activity (Sylvia *et al.*, 1993).

Once they are produced, matrix vesicles are probably under autocrine regulation by chondrocytes. Evidence to support autocrine regulation would include the fact that both resting zone and growth zone cells produce 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ constitutively (Schwartz *et al.*, 1992a). This production is regulated by 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ themselves, as well as by TGF-β and dexamethasone. When isolated matrix vesicles are incubated directly with 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$, the same effects on alkaline phosphatase and phospholipase A_2 noted in intact cultures are observed (Schwartz *et al.*, 1988b), indicating that at least some of

the response is due to nongenomic actions of the hormone directly on the matrix vesicle. Under these same experimental conditions, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ alter matrix vesicle membrane fluidity, and the effect is both metabolite-specific and cell maturation-dependent (Swain *et al.*, 1993).

Changes in membrane fluidity may result from the physical presence of the hormone, as seen when cholesterol or cholesterol derivatives are incorporated into phospholipid bilayers (Rasmussen *et al.*, 1982; Farley *et al.*, 1985; Husain *et al.*, 1990). They may also result from downstream changes in the length and saturation of phospholipid fatty acid residues (Pollesello, 1990). Other studies have shown that both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ regulate phospholipid metabolism (Schwartz *et al.*, 1990; Swain *et al.*, 1992), including an effect of 1,25-$(OH)_2D_3$ on phospholipase C (Sylvia *et al.*, 1993).

G. Vitamin D and Protein Kinase C

Sylvia and co-workers recently showed that the vitamin D metabolites regulate protein kinase C activity in a metabolite-specific, cell maturation-dependent manner (Sylvia *et al.*, 1993). 1,25-$(OH)_2D_3$ stimulates PKC in growth zone chondrocytes but has no effect on resting zone cells. 24,25-$(OH)_2D_3$ stimulates activity in resting zone chondrocytes but has no effect on growth zone cells. Both metabolites promote translocation of protein kinase C to the plasma membrane in their respective target cells.

The protein kinase C family of isoenzymes mediate transduction of extracellular signals to intracellular effectors and serve critical functions in growth and differentiation (Inoue and Nishizuka, 1977; Ohno *et al.*, 1991; Hug and Sarre, 1993). Protein kinase C alpha, beta and gamma are calcium-dependent, phospholipid-dependent isoforms; delta and epsilon are calcium-

independent, phospholipid-dependent isoforms; and zeta is calcium-independent and diacyglycerol-independent (Liyanage *et al.*, 1992). Relatively little is known about protein kinase C activation and regulation in bone and cartilage cells. There is some evidence that suggests that PKC is involved in vitamin D signaling in bone. Protein kinase C inhibition was shown to block 1,25-$(OH)_2D_3$-induced osteocalcin production in osteoblasts (van Leeuwen *et al.*, 1992). These results would support a protein kinase C-dependent mechanism for 1,25-$(OH)_2D_3$ signaling in bone. 1,25-$(OH)_2D_3$ increases inositol trisphosphate and diacylglycerol levels in osteoblasts (Civitelli *et al.*, 1990) suggesting a phospholipase C-dependent mechanism. Additionally, 1,25-$(OH)_2D_3$ can activate Ca^{++} channels in the intestine in a process referred to as "transcaltachia" (de Boland and Norman, 1990), and protein kinase C is known to activate Ca^{++} channels (Yamaguchi *et al.*, 1987).

1,25-$(OH)_2D_3$ exerts its effects on protein kinase C activity in growth zone chondrocytes through nongenomic mechanisms, and the process involves phospholipase C (Sylvia *et al.*, 1993). In contrast, 24,25-$(OH)_2D_3$ appears to act through classic steroid hormone receptor mechanisms in resting zone chondrocyte cultures. It is likely that 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ have distinct functions in cartilage and transduce their transmembrane signals via distinct pathways. A primary function of 1,25-$(OH)_2D_3$ is to promote calcium transport (Lieberherr, 1989), whereas 24,25-$(OH)_2D_3$ appears to be involved in promoting early differentiation events (de Boland and Nemere, 1992). Thus, a phospholipase C-dependent mechanism, with consequent calcium transport, is not a feature of the 24,25-$(OH)_2D_3$-dependent protein kinase C activation.

While synthesis of matrix vesicles may be under genomic control, it is likely that cells may use nongenomic regulatory mechanisms for controlling events in the extracellular matrix. Resting zone and growth zone chondrocytes synthesize, secrete and respond to vitamin D metabolites in a

metabolite-specific, membrane-specific and cell maturation-specific manner, suggesting that the secreted hormones may interact directly with both matrix vesicle and plasma membranes. The mechanisms used by chondrocytes to discriminate the response of matrix vesicles to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ from the response of the intact cell to these hormones, however, are unclear.

Since matrix vesicles are extracellular and contain no DNA or RNA, they represent a unique model for studying nongenomic mechanisms of hormone action. The purpose of the present study was to determine whether 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ exert their nongenomic effects this organelle by regulating matrix vesicle protein kinase C activity. To do this, we determined whether protein kinase C activity is present in matrix vesicles, whether there is a differential distribution of enzyme species between the plasma and matrix vesicle membranes, and whether the matrix vesicle is regulated by 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in a metabolite-specific, cell maturation-dependent manner.

II. MATERIALS AND METHODS

A. Cell Culture

The cell culture system that was used compares chondrocytes at two distinct stages in the endochondral differentiation pathway (Boyan et al., 1988b). Ribcages were removed from 125g Sprague-Dawley rats and placed in Dulbecco's modified Eagle's medium (DMEM). The resting zone and adjacent growth zone cartilage were dissected and the intervening tissue removed to limit cross-contamination of cell zones. The cartilage was then sliced and incubated overnight in DMEM containing antibiotics at 37°C with 5% CO_2 and 100% humidity. The DMEM was replaced by two 20-minute washes in Hanks' balanced salt solution (HBSS), followed by sequential incubations in 1% trypsin for one hour and 0.02% collagenase (Type II, GIBCO, Grand Island, NY) for three hours. After enzymatic digestion, cells were separated from debris by filtration, collected by centrifugation at 500 x g for ten minutes, resuspended in DMEM, and plated at a density of 10,000 cells/cm^2 for resting zone cells or 25,000 cells/cm^2 for growth zone cells. Cultures were incubated in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-fungizone, and 50μg/ml sodium ascorbate in an atmosphere of 5% CO_2 at 37°C and 100% humidity. The media were changed at 24 hours and then at 72-hour intervals. At confluence (7-10 days), cells were subcultured using the same plating densities and technique as those described above and allowed to return to confluence. For all experiments, third passage, confluent cultures were subpassaged into either 24-well plates or T-25 flasks and grown to confluence. Cells were subcultured in this manner since previous studies have demonstrated a retention of differential phenotypic markers (Boyan *et al.*, 1988a and b; Dean *et al.*, 1992; Schwartz *et al.*, 1992a).

B. Membrane Isolation

Plasma membranes and matrix vesicles were prepared as previously described (Boyan *et al.*, 1988b). At harvest, cultures were trypsinized and the cells collected by centrifugation. Plasma membranes were isolated by differential centrifugation and sucrose density centrifugation of cell homogenates. Matrix vesicles were isolated by differential centrifugation of the trypsin digest supernatant. Membrane fractions were resuspended in 1.0 ml 0.9% NaCl and stored at -70°C until used. Matrix vesicles isolated from two T-75 flasks were combined before resuspension and storage. Plasma membranes from each culture were stored independently. Since homogenization or lysing the cultures is not necessary for matrix vesicle isolation, the matrix vesicles are intact and rightside out after isolation. They contain no DNA or RNA; therefore, any effect of hormones directly on the matrix vesicles will not involve new gene transcription or translation.

C. Preparation of Cell Layer Lysates

Lysates of fourth passage chondrocyte cultures grown in 24-well plates were prepared by washing the cultures once with 0.5ml ice-cold PBS followed by a 30 minute lysis in in 0.3ml RIPA buffer [20mM Tris-HCl, pH 7.5, 150mM NaCl, 5mM ethylenediamine-tetraacetic acid (EDTA), 1mM phenylmethylsulfonylfluoride (PMSF), and 1% NP-40 detergent (Sigma Chemical Corp., Fairlawn, NJ)].

D. Protein Kinase C Assay

Protein kinase C specific activity was measured in chondrocyte culture lysates, matrix vesicles or plasma membrane preparations. For all experiments, protein content was determined by the BCA method (Smith *et al.*, 1985) using duplicate 25 µl samples of membrane suspension or culture lysate. For measurement of enzyme activity, samples (35µl) were incubated for 20

minutes with a lipid preparation (5μl) containing 0.3 mg/ml phosphatidylserine, 10 μM phorbol-12-myristate-13-acetate, and Triton X-100 mixed micelles, which provides the necessary cofactors and conditions for optimal activity (Bell *et al.*, 1986). To this mixture, a high-affinity myelin basic protein peptide (MBP, 8μM), (GIBCO-BRL Grand Island, NY) and ^{32}P-ATP (25μ Ci/ml) were added to a final assay volume of 50μl. Following a ten-minute incubation in a 30°C waterbath, samples were spotted onto phosphocellulose discs, which were then washed twice with 1% phosphoric acid and once with distilled water to remove unincorporated label prior to placement in a scintillation counter. The phosphocellulose disc strongly binds the MBP peptide substrate, which becomes ^{32}P-labeled during the protein kinase C reaction, and unincorporated ^{32}P-ATP is washed free of the discs during the phosphoric acid wash step.

E. Direct Effect of PKC Activator and Inhibitor

Matrix vesicles or plasma membrane suspensions (30μl) were incubated for either 9 or 90 minutes at 37°C in the absence or presence of 5μl 0.9% saline containing bryostatin-1 (Warren *et al.*, 1988) or pseudosubstrate inhibitor peptide (Yasuda *et al.*, 1990) so that the final concentration of bryostatin-1 was 10^{-9}M-10^{-8}M and that of the inhibitor peptide was 10^{-6}M-10^{-5}M. Samples were then assayed for protein kinase C activity. Bryostatin-1 was obtained from Bristol-Myers Squibb (Wallingford, CT) and a stock solution in dimethyl sulfoxide (DMSO) was diluted in 0.9% NaCl prior to use. Control samples received the same concentration of DMSO in 0.9% saline as the bryostatin-treated samples. Pseudosubstrate inhibitor peptide was from GIBCO-BRL (Grand Island, NY); it was supplied in a buffered solution and was diluted in 0.9% NaCl before use.

F. Determination of Protein Kinase C Isoforms

Culture lysates, matrix vesicles or plasma membranes (0.3 ml of the 1 ml preparations) from resting zone or growth zone cell cultures were incubated on ice for 1 hour with 6μl of a 1:10 dilution of non-specific rabbit IgG1 or isoform-specific anti-protein kinase C (PKC) rabbit IgG1 in 0.9% saline, resulting in a final antibody dilution of 1:500. Protein G-agarose (20μl) (Oncogene Science, Inc., Uniondale, NY) was added for 4 hours to clear the samples of immunoreactive PKC isoforms and any remaining unbound antibody. Following precipitation of this material, 35μ l of the supernatant was assayed for PKC activity. The following antibodies were used: polyclonal rabbit antibodies specific for the alpha, beta, delta, epsilon and zeta isoforms were obtained from GIBCO-BRL (Grand Island, NY), and the non-specific rabbit IgG1 was obtained from Sigma Chemical Corp. (Fairlawn, NJ).

Western blots were used to verify that protein kinase C was present in the chondrocyte membrane fractions. Isolated matrix vesicle and plasma membrane preparations (10μg protein in 1ml 0.9% NaCl) were incubated for one hour with 2μl of either a monoclonal pan-specific anti-PKC IgG (1:500) (Amersham Corp., Arlington Heights, IL), or polyclonal anti-PKCζ (1:500) (GIBCO-BRL, Grand Island, NY), followed by a four-hour incubation with 20μl of protein G-agarose. In a few experiments, samples were pre-incubated with protein G-agarose prior to incubation with antibodies in order to remove any proteins which might bind nonspecifically. The supernatant was then incubated with antibody and any immune-reactive protein was precipitated with 20μl protein G-agarose. The immunoprecipitated pellets were washed twice with RIPA buffer and twice with ultrapure deionized water. Prior to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the immunoreacted protein was dissociated by heating for 6 minutes at 100°C. Samples were electrophoresed on a 12% gel for 16 hours at 10-20 mA).

Authentic purified rat brain protein kinase C (Calbiochem, San Diego, CA) was run alongside the experimental samples to facilitate determination of the chondrocyte protein kinase C band(s). The gels were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a semi-dry electroblotter (Hoefer Scientific, San Francisco, CA) (2.5 mA/cm^2 for 1.5 hours). The membranes were then blocked overnight in 5% nonfat dry milk and probed with an anti-PKC primary antibody (1:1250 dilution) for one hour. This was followed by horseradish peroxidase-conjugated anti-IgG secondary antibody (1:2000 dilution) for one hour. Each antibody was diluted in PBS-0.1% Tween-20 (PBST), and the membranes were washed three times with PBST before and after addition of the secondary antibody. Visualization of the protein kinase C bands was achieved using the Renaissance chemiluminescence reagent (DuPont-NEN, Boston, MA) for one minute. The membrane was then exposed to Kodak XAR-5 film and the film developed.

G. Immunoelectron Microscopic Localization of Protein Kinase C

The presence of protein kinase C in matrix vesicles was verified by immunogold labelling and transmission electron microscopy. Briefly, matrix vesicles isolated from confluent, fourth passage resting zone cultures were embedded in LR Gold (Polysciences, Inc., Warrington, PA) under ultraviolet light for 20 hours, sectioned and placed on carbon coated nickel grids. Sections were treated with PBS containing either normal rabbit IgG_1, or PBS containing either pan-specific anti-PKC antibody or anti-PKCζ antibody (1:500 dilution) for 15 minutes, washed with 0.1M Tris-HCl, pH 7.5, followed by a gold-labeled secondary antibody (1:5 in 0.1M Tris-HCl plus 1% bovine serum albumin) for 30 minutes, rinsed again and fixed in 2% glutaraldehyde in 0.1M PBS for 20 min. The grids were dried and stained with uranyl acetate prior to electron microscopy.

H. Effect of Lipid

To test the lipid dependence of matrix vesicle and plasma membrane protein kinase C, 35µl samples were incubated for 90 minutes at 37°C with either 5µl RIPA or 5µl lipid preparation (described above). Samples were then assayed for enzyme activity.

I. Effect of Vitamin D_3 Metabolites

The direct effects of 1,25-$(OH)_2D_3$ and of 24,25-$(OH)_2D_3$ on protein kinase C activity were tested using matrix vesicles and plasma membranes isolated from confluent, fourth passage cultures of growth zone or resting zone chondrocytes, respectively, as described previously (Boyan *et al.*, 1988b; Schwartz *et al.*, 1988b). Matrix vesicles or plasma membranes in 1ml 0.9% NaCl containing 10% FBS were incubated in the absence (vehicle only) or presence of a final concentration of 10^{-9}M-10^{-8}M 1α,25-$(OH)_2D_3$ or 10^{-8}M-10^{-7}M 24R,25-$(OH)_2D_3$ (gifts of Dr. Milan Uskokovic, Hoffman La-Roche, Nutley, NJ) for either 9, 90, or 270 minutes at 37° C. Following incubation with hormone, samples were assayed for protein kinase C activity (35µl) or protein content (25µl).

To determine which isoform was responsible for any vitamin D effect, membrane suspensions were incubated with anti-PKCα or anti-PKCζ antibody (1:500 final dilution) after incubation with 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$. Immunoreactive PKC was precipitated with protein G-agarose and protein kinase C activity remaining in the supernatant measured. Titration of the anti-isoform antibodies determined that a final dilution of 1:500 was the appropriate concentration, since higher antibody doses did not further reduce PKC activity in either matrix vesicles or plasma membranes.

To further define the vitamin D-dependent protein kinase C isoform(s) in matrix vesicles and plasma membranes, antibody neutralization studies were performed. Antibodies specific for

the α and ζ isoforms were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Matrix vesicles or plasma membranes isolated from resting zone chondrocyte cultures (30μl) were incubated for one hour with 2μl RIPA buffer containing anti-isoform antibody (1:500 final dilution) in the presence or absence of 10^{-8}M 24,25-$(OH)_2D_3$. Protein G-agarose (20μl) (Oncogene Science, Inc., Uniondale, NY) was added as described above, samples centrifuged for one minute at 15,000 X g, and 35μl of the supernatant mixed with 5μl of lipid preparation and assayed for PKC activity. Controls consisted of incubating the membrane fractions with 2μl of one of the following: RIPA buffer alone; nonspecific IgG; or antibody (1:500 dilution).

J. Effect of Protease Inhibitors

Matrix vesicles isolated from confluent, fourth passage resting zone chondrocytes (30μl) were incubated with 10μl RIPA buffer (which contains 1mM PMSF) or with 10μl RIPA buffer plus 1 ng/ml leupeptin and 0.1μg/ml aprotinin (Protease Inhibitor Cocktail = PIC buffer) at 37°C for 9, 90, or 270 minutes. Following incubation, 10μl of PKC substrate solution prepared either in RIPA buffer or PIC buffer was added, and protein kinase C activity was assayed as described above.

K. Statistical Analysis

Protein kinase C specific activity data are expressed as the mean pMol phosphate transferred/μg protein/minute ± standard error of the mean (SEM), N=6. For experiments using culture lysates, each N equals one culture. For experiments using membrane preparations, each N represents the combined membranes of two cultures. Observations were validated by repeating each experiment a minimum of two times. Significance between treatment and controls was determined by Bonferroni's t-test using $P < 0.05$ confidence limits.

III. RESULTS

Protein kinase C activity was detected in isolated matrix vesicles from both growth zone and resting zone cell cultures (Figures 1A and 1B). Although basal levels (9 minutes) were higher in matrix vesicles produced by the growth zone chondrocytes in the experiment shown, this was not a consistent observation. There was a time-dependent loss of protein kinase C activity in matrix vesicles from both types of cultures. Over all time course experiments done (N=6 for growth zone cell cultures, N=8 for resting zone cell cultures), the loss of specific activity during the first 90 minutes was greater in matrix vesicles produced by growth zone cells (50.9 + 3.8%) than those from resting zone cells (28.9 + 5.3%). By 270 minutes, the percent loss of activity was comparable in both types of matrix vesicles (56.2 + 0.6% v. 54.2 + 3.2%).

The protein kinase C activator, bryostatin-1, produced a dose-dependent increase in matrix vesicle kinase activity (Figure 1A). At 9 minutes, 10^{-9}M bryostatin-1 increased kinase activity by 41.1% in matrix vesicles produced by growth zone cells and by 108.7% in matrix vesicles produced by resting zone cells. 10^{-8}M bryostatin-1 further increased kinase activity in matrix vesicles isolated from both resting zone and growth zone cell cultures. After 90 minutes, bryostatin-1 had no effect on kinase activity in matrix vesicles produced by growth zone chondrocytes, while enzyme activity in matrix vesicles from the resting zone cell cultures was still at increased levels.

Matrix vesicle protein kinase C activity was inhibited by a specific pseudosubstrate inhibitor peptide (Figure 1B). At 9 minutes, 10^{-6}M inhibitor peptide reduced kinase activity by 45.5% in matrix vesicles produced by growth zone cells and by 21.0% in matrix vesicles produced by resting zone cells. 10^{-5}M inhibitor peptide further decreased kinase activity in matrix vesicles

Figure 1A. EFFECT OF BRYOSTATIN-1 ON CHONDROCYTE MATRIX VESICLE PROTEIN KINASE C ACTIVITY. Matrix vesicles isolated from fourth passage growth zone (GC) and resting zone (RC) costochondral chondrocytes were treated with 10^{-9}-10^{-8}M bryostatin-1 for 9 or 90 minutes and then assayed for protein kinase C (PKC) specific activity. Data represent the mean $\pm$ SEM of PKC activity in matrix vesicles (MV) from a representative experiment; N=6. *$P<0.05$, treatment vs. control.

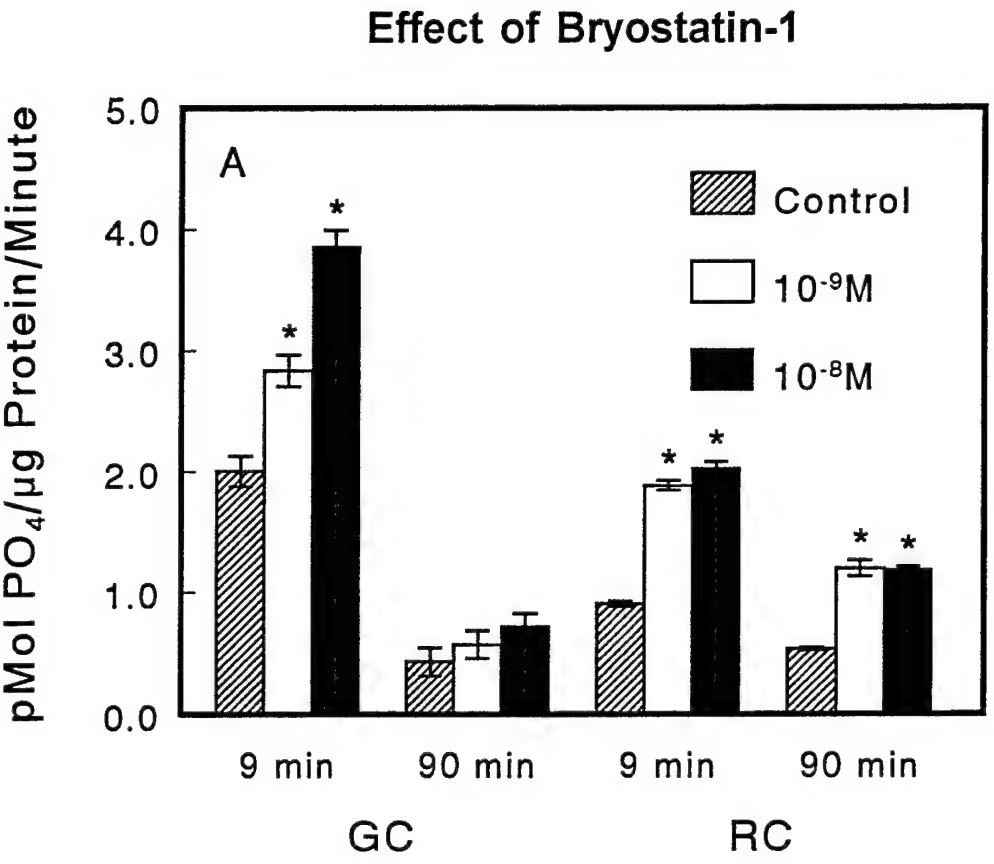
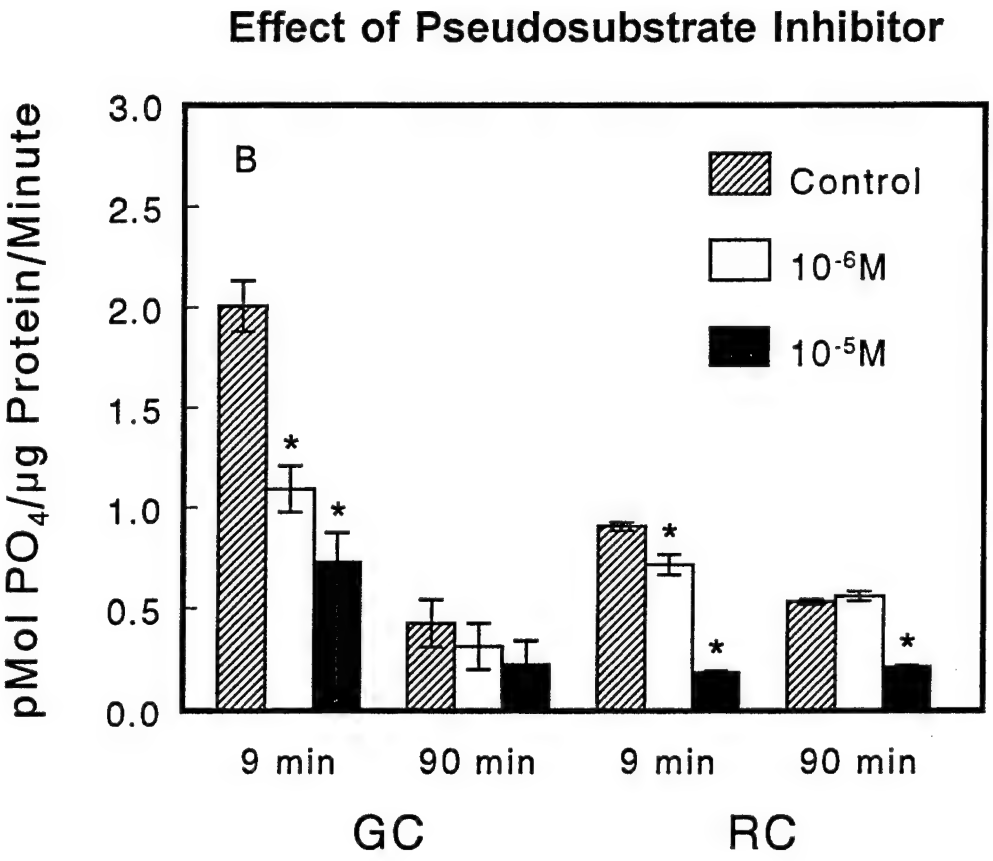

Effect of Bryostatin-1
A
Control
10-9M
10-8M
pMol PO4/µg Protein/Minute
5.0
4.0
3.0
2.0
1.0
0.0
9 min
90 min
9 min
90 min
GC
RC

Figure 1B. EFFECT OF PSEUDOSUBSTRATE INHIBITOR PEPTIDE ON CHONDROCYTE MATRIX VESICLE PROTEIN KINASE C ACTIVITY. Matrix vesicles isolated from fourth passage growth zone (GC) and resting zone (RC) costochondral chondrocytes were treated with 10^{-6}-10^{-5}M pseudosubstrate inhibitor peptide for 9 or 90 minutes and then assayed for protein kinase C (PKC) specific activity. Data represent the mean $\pm$ SEM of PKC activity in matrix vesicles (MV) from a representative experiment; N=6. *$P<0.05$, treatment vs. control.


Effect of Pseudosubstrate Inhibitor
B
Control
10-6M
10-5M
pMol PO4/µg Protein/Minute
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
*
9 min
90 min
9 min
90 min
GC
RC

from both cell types. Significant inhibition of kinase activity in matrix vesicles produced by growth zone cells was only observed at 9 minutes, but not at 90 minutes. In contrast, resting zone cell matrix vesicle kinase activity was significantly inhibited at 90 minutes, but only by 10^{-5}M pseudosubstrate inhibitor peptide.

Western blots demonstrated the presence of protein kinase C in matrix vesicles and plasma membranes from both growth zone and resting zone chondrocytes (Figure 2). It was necessary to preclear the samples by incubation with Protein G-agarose (lanes 3-6), to remove non-specific proteins, in order to obtain a definitive signal at 77 kD that co-migrated with the authentic purified protein kinase C standard (lanes 2 and 7). This band was later identified in other experiments, in which pan-specific antibody immunoprecipitates were probed with an anti-PKCα isoform-specific antibody, as PKCα (data not shown). When immunoprecipitation was performed without preclearing (lanes 8-11), a doublet was observed at 77 kD, along with a second band at 68 kD. The 77 kD band dominated in plasma membranes (lanes 9 and 11). Immunoprecipitates of matrix vesicles incubated with anti-PKCζ antibody demonstrated that the 68 kD band was PKCζ (lanes 12 and 13).

The dominant protein kinase C isoform present in chondrocyte cell culture lysates, as well as inisolated plasma membranes, was PKCα, based on the inhibition of enzyme activity by anti-PKC α antibody (Table I, Figures 3B and 4B). When compared to the non-specific IgG control, only anti-PKCα antibody significantly decreased protein kinase C activity in resting zone chondrocyte culture lysates and plasma membranes, inhibiting activity by 29.3% and 71.6%, respectively (Table I, Figure 3B). Anti-PKCα antibody did not reduce protein kinase C activity in the matrix vesicles. In contrast, anti-PKCζ reduced kinase activity by 66.9% in matrix vesicles from resting

Figure 2. WESTERN BLOT OF PROTEIN KINASE C ISOFORMS IN MATRIX VESICLES (MV) AND PLASMA MEMBRANES (PM) ISOLATED FROM GROWTH ZONE (GC) AND RESTING ZONE (RC) CHONDROCYTE CULTURES. Lane 1: enhanced chemiluminescence molecular weight markers (Amersham Corp., Arlington Heights, IL). Lanes 2-6: gel in which lane 2 is the PKC standard and lanes 3-6 are samples which were treated with Protein G-agarose prior to immunoprecipitation as described in Methods (lane 3, GC MV; lane 4, GC PM; lane 5, RC MV; lane 6, RC PM). Lanes 7-11: gel in which lane 7 is the PKC standard and lanes 8-11 are samples which were immunoprecipitated as described in Methods (lane 8, GC MV; lane 9, GC PM; lane 10, RC MV; lane 11, RC PM). Lanes 12-13 is a gel in which GC MV (lane 12) and RC MV (lane 13) were immunoprecipitated with anti-PKCζ antibody.

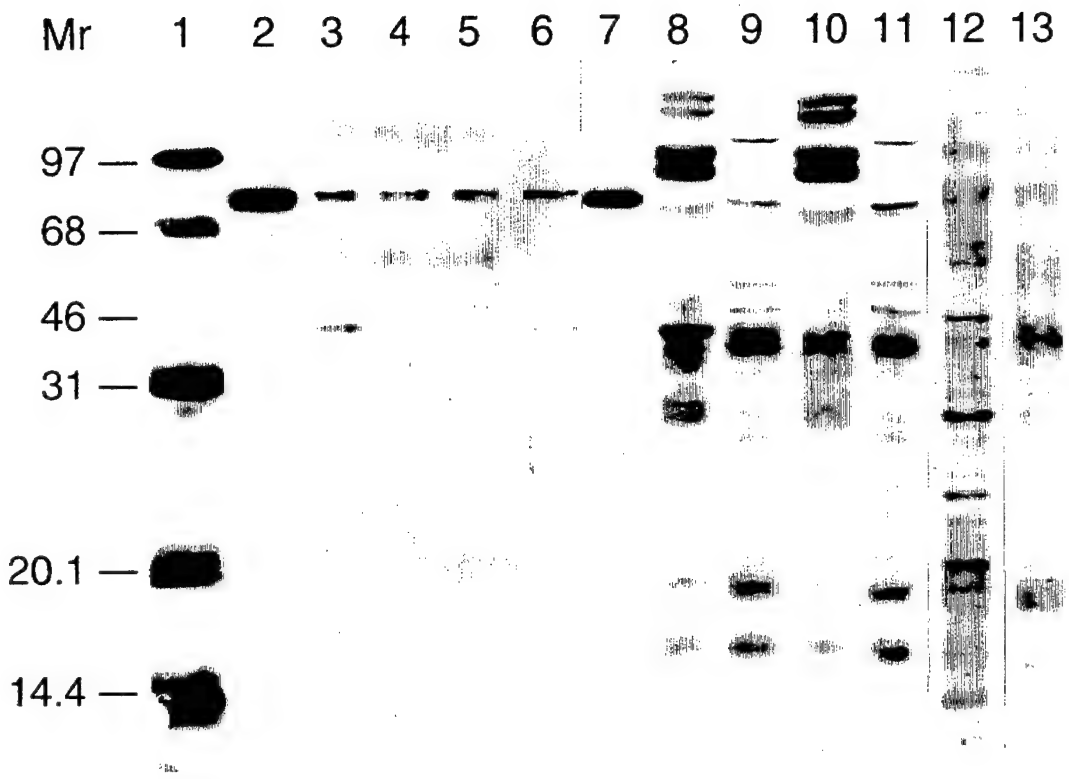
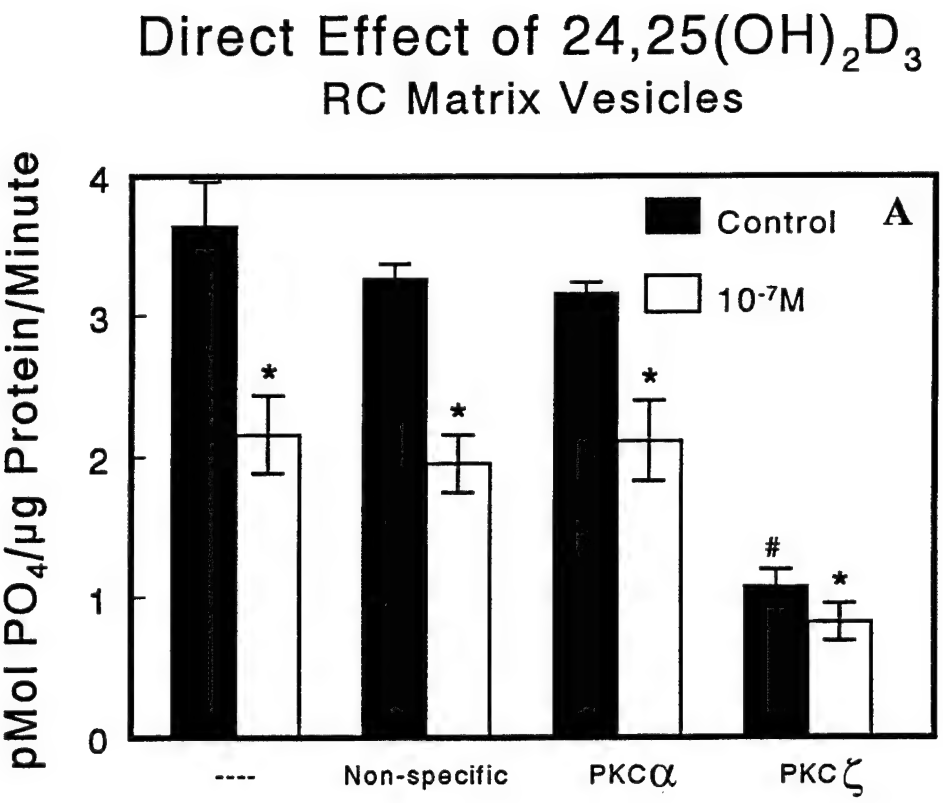

Mr
1
2
3
4
5
6
7
8
9
10
11
12
13
97
68
46
31
20.1
14.4

Table 1. EFFECT OF ISOFORM-SPECIFIC ANTIBODIES ON PROTEIN KINASE C SPECIFIC ACTIVITY OF RESTING ZONE COSTOCHONDRAL CHONDROCYTE FRACTIONS.

	Protein Kinase C Specific Activity (pMol PO_4/μg protein/min)		
Antibody	**Cell Culture Lysate**	**Matrix Vesicles**	**Plasma Membranes**
None	0.34 ± 0.09	1.82 ± 0.02	2.80 ± 0.64
Non-specific IgG	0.24 ± 0.03	1.63 ± 0.01	2.68 ± 0.07
Alpha	0.17 ± 0.08*	1.58 ± 0.04	0.80 ± 0.01*
Beta	0.25 ± 0.04	1.62 ± 0.03	2.53 ± 0.03
Delta	0.25 ± 0.09	1.64 ± 0.00	2.56 ± 0.04
Epsilon	0.27 ± 0.11	1.57 ± 0.05	2.86 ± 0.03
Zeta	0.22 ± 0.10	0.54 ± 0.06*	2.41 ± 0.04

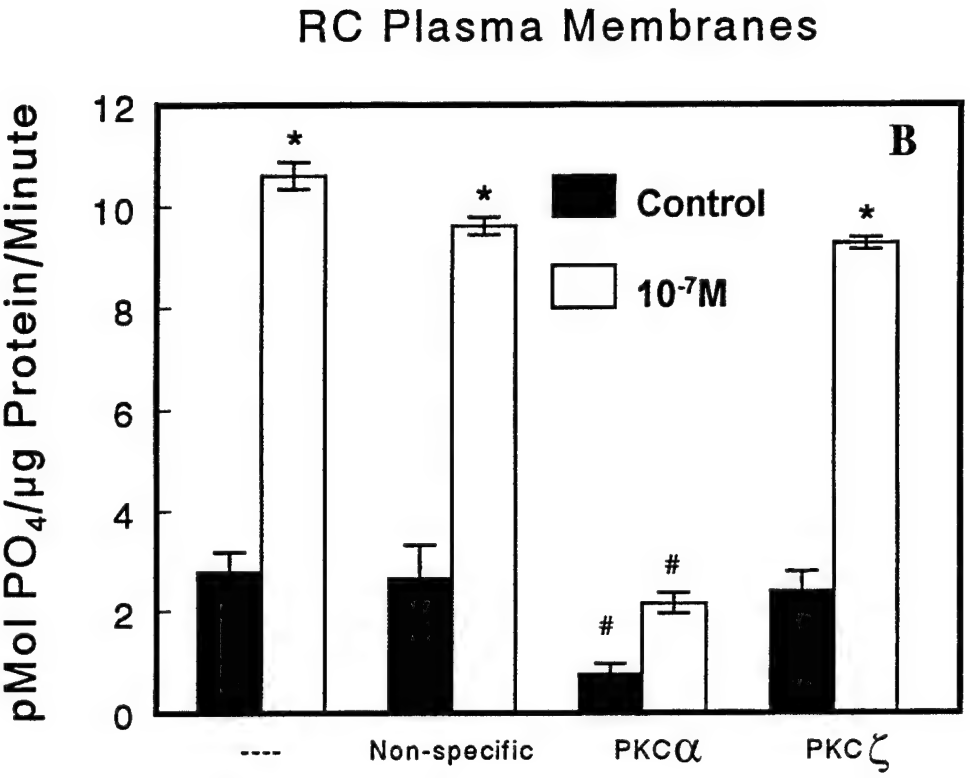
Residual protein kinase C specific activity (pMol PO_4/μg protein/min) of resting zone chondrocyte fractions following treatment with anti-protein kinase C isoform-specific antibodies. Values represent the mean ± SEM for six independent cultures or membrane fractions. *$P<0.05$, specific antibody vs. nonspecific IgG.

Figure 3A. EFFECT OF ANTI-PKC ISOFORM-SPECIFIC ANTIBODIES ON PKC ACTIVITY IN MATRIX VESICLES (MV) ISOLATED FROM THE CELL LAYER OF RESTING ZONE CHONDROCYTES (RC). MV were isolated, incubated with control medium or medium containing 10^{-7}M $24,25(OH)_2D_3$ for 90 minutes at 37°C, followed by a second incubation for 60 minutes on ice with or without (control = no added IgG; non-specific = rabbit anti-mouse IgG) anti-PKC isoform-specific antibodies, and then assayed. Data represent mean ± SEM of PKC activity remaining in the supernatant. *P < 0.05, control vs. 24,25-$(OH)_2D_3$-treated media; #P < 0.05, vs. no added IgG.


Direct Effect of 24,25(OH)2D3
RC Matrix Vesicles
pMol PO4/µg Protein/Minute
0
1
2
3
4
Control
10-7M
A
*
*
*
#
*

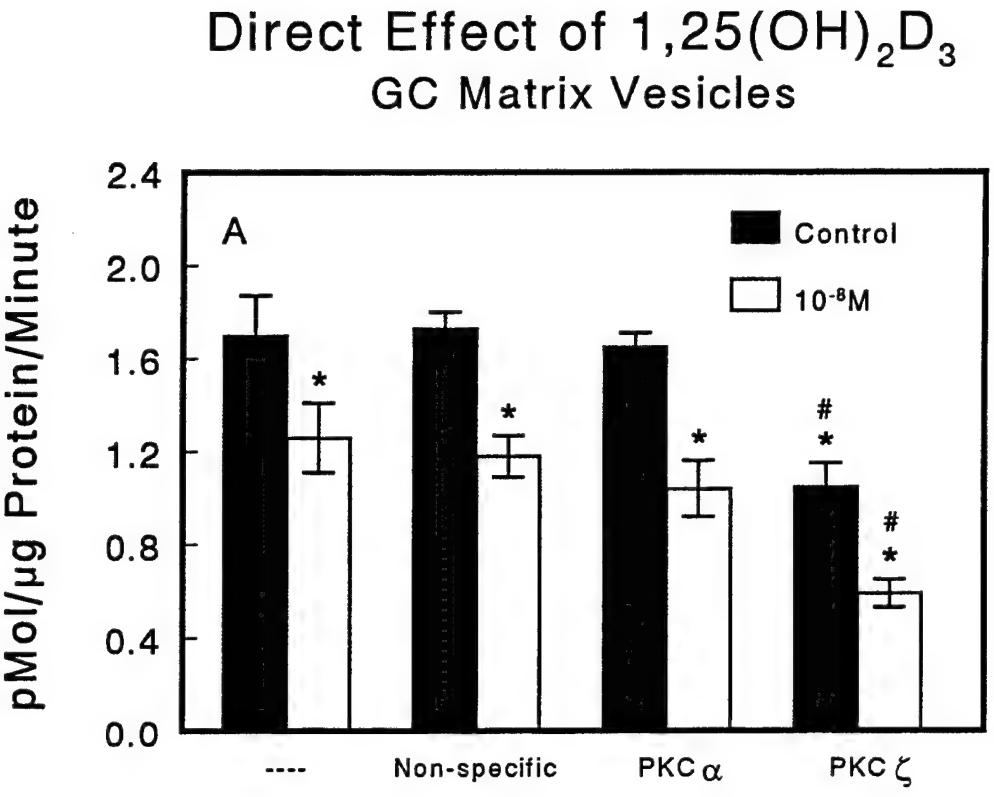
Non-specific
PKCα
PKCζ

Figure 3B. EFFECT OF ANTI-PKC ISOFORM-SPECIFIC ANTIBODIES ON PKC ACTIVITY IN PLASMA MEMBRANES (PM) ISOLATED FROM THE CELL LAYER OF RESTING ZONE CHONDROCYTES (RC). PM were isolated, incubated with control medium or medium containing 10^{-7}M $24,25(OH)_2D_3$ for 90 minutes at 37°C, followed by a second incubation for 60 minutes on ice with or without (control = no added IgG; non-specific = rabbit anti-mouse IgG) anti-PKC isoform-specific antibodies, and then assayed. Data represent mean $\pm$ SEM of PKC activity remaining in the supernatant. *$P < 0.05$, control vs. $24,25-(OH)_2D_3$-treated media; $^{\#}P < 0.05$, vs. no added IgG.


RC Plasma Membranes
B
pMol PO4/µg Protein/Minute
12
10
8
6
4
2
0
Control
10-7M
*
*
*
#
#

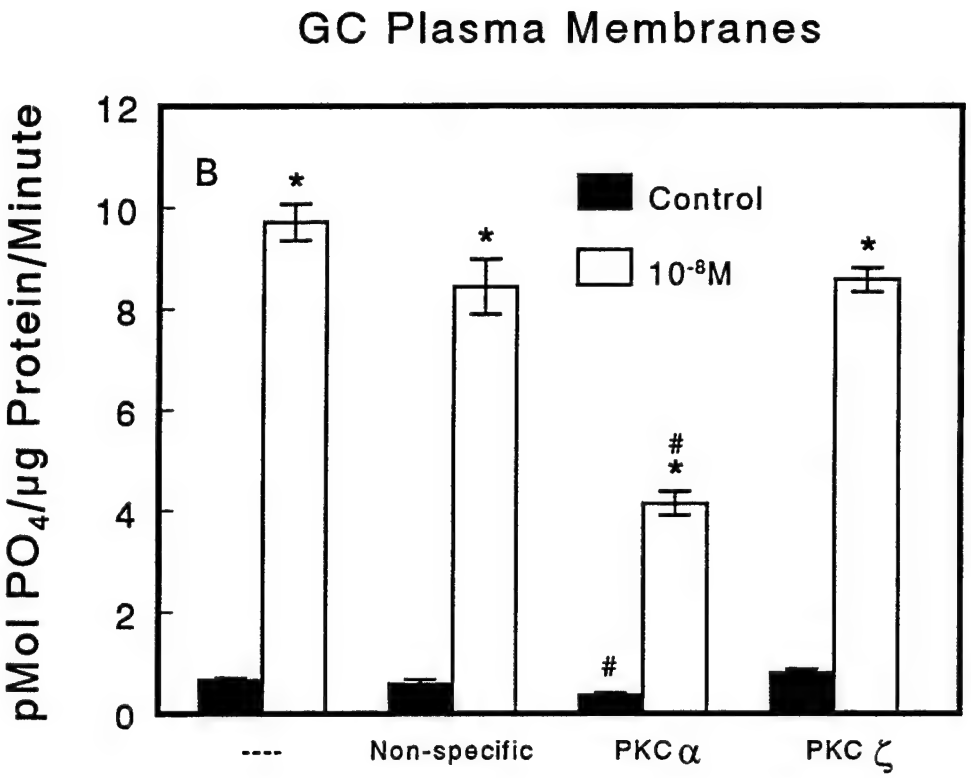
Non-specific
PKCα
PKCζ

Figure 4A. EFFECT OF ANTI-PKC ISOFORM-SPECIFIC ANTIBODIES ON PKC ACTIVITY IN MATRIX VESICLES (MV) ISOLATED FROM GROWTH ZONE CHONDROCYTE CULTURES (GC). MV were isolated, incubated with control medium or medium containing 10^{-8}M 1,25-$(OH)_2D_3$ for 9 minutes at 37°C, incubated without (control = no added IgG; non-specific = rabbit anti-mouse IgG) or with anti-PKC isoform-specific antibodies, and then assayed. Data represent the mean ± SEM of PKC activity remaining in the supernatant. *P < 0.05, control vs. 1,25-$(OH)_2D_3$-treated media; #P <0.05, vs. no added IgG.


Direct Effect of 1,25(OH)2D3
GC Matrix Vesicles
pMol/µg Protein/Minute
2.4
2.0
1.6
1.2
0.8
0.4
0.0
A
Control
10-8M
*
*
*
#
*
#
*

Non-specific
PKC α
PKC ζ

Figure 4B. EFFECT OF ANTI-PKC ISOFORM-SPECIFIC ANTIBODIES ON PKC ACTIVITY IN PLASMA MEMBRANES (PM) ISOLATED FROM GROWTH ZONE CHONDROCYTE CULTURES (GC). PM were isolated, incubated with control medium or medium containing 10^{-8}M 1,25-$(OH)_2D_3$ for 9 minutes at 37°C, incubated without (control = no added IgG; non-specific = rabbit anti-mouse IgG) or with anti-PKC isoform-specific antibodies, and then assayed. Data represent the mean ± SEM of PKC activity remaining in the supernatant. *P < 0.05, control vs. 1,25-$(OH)_2D_3$-treated media; #P <0.05, vs. no added IgG.


GC Plasma Membranes
B
pMol PO4/µg Protein/Minute
12
10
8
6
4
2
0
Control
10-8M
*
*
*
*
#
#

Non-specific
PKC α
PKC ζ

zone cell cultures (Table I, Figure 3A).

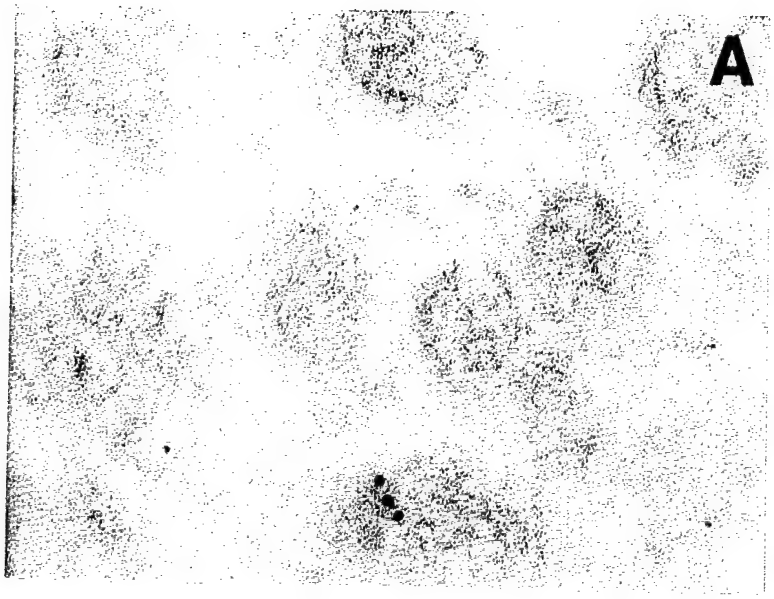
Similar results were obtained for membrane fractions from growth zone chondrocyte cultures. Anti-PKCζ reduced enzyme activity by 39.3% in matrix vesicles isolated from growth zone cell cultures (Figure 4A), with only minimal effects upon cell culture lysate (data not shown) or plasma membrane (Figure 4B) kinase activity. Anti-PKCα inhibited protein kinase C in cell culture lysates by 42.3% (data not shown) and in isolated plasma membranes by 38.3% (Figure 4B).

Electron microscopy using immunogold labeling with pan-specific antibody verified the presence of PKC in matrix vesicles isolated from cultures of growth zone chondrocytes, as well as from resting zone cell cultures (data not shown). While all matrix vesicles in the field did not exhibit reactivity to the PKC antibody, the majority were positive. When matrix vesicle preparations were labeled using normal rabbit IgG1 (Figure 5A), there was essentially no nonspecific binding. However, when sections were incubated with anti-PKCζ antibody (Figure 5B), the majority of the vesicles were positive for PKCζ.

The PKC specific activity in plasma membranes was lipid-dependent, while that in the matrix vesicles was not. PKC activity in plasma membranes isolated from resting zone chondrocytes was reduced by 83.4% when lipid was not included in the assay (Figure 6). In contrast, PKC activity in matrix vesicles isolated from resting zone cell cultures was reduced by only 9.3%, while that of matrix vesicles isolated from growth zone cell cultures was only reduced by 1.4% in the absence of lipid.

Protein kinase C activity was directly regulated by vitamin D metabolites in a membrane-specific manner. PKC in matrix vesicles isolated from growth zone cell cultures was inhibited by

Figure 5. TRANSMISSION ELECTRON MICROGRAPH SHOWING IMMUNOGOLD LABELING OF PKCζ IN MATRIX VESICLES ISOLATED FROM CONFLUENT, FOURTH PASSAGE RESTING ZONE CULTURES. Samples were embedded in LR Gold, sectioned, treated with either non-specific rabbit IgG1 (Panel A) or anti-PKCζ specific antibody (Panel B) followed by a gold-labeled secondary antibody and visualized by electron microscopy. Bar = 0.1 μm; magnification = 150,000x.


A

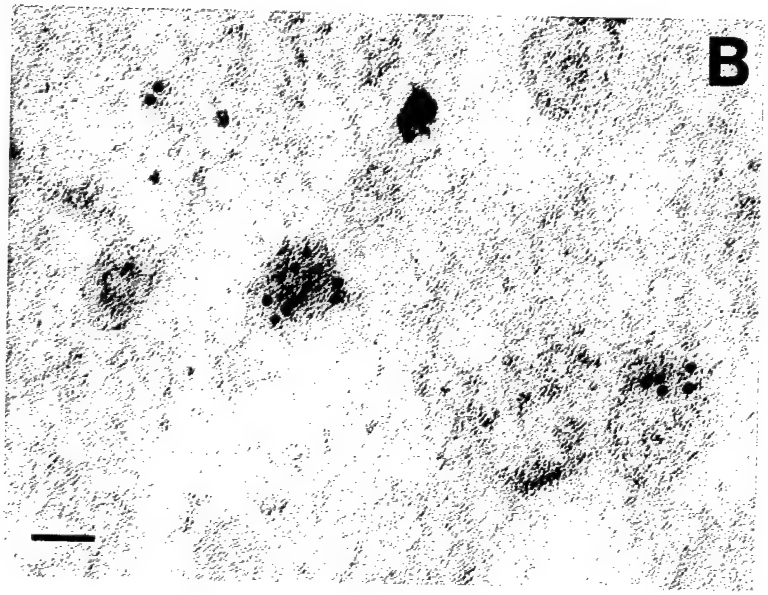
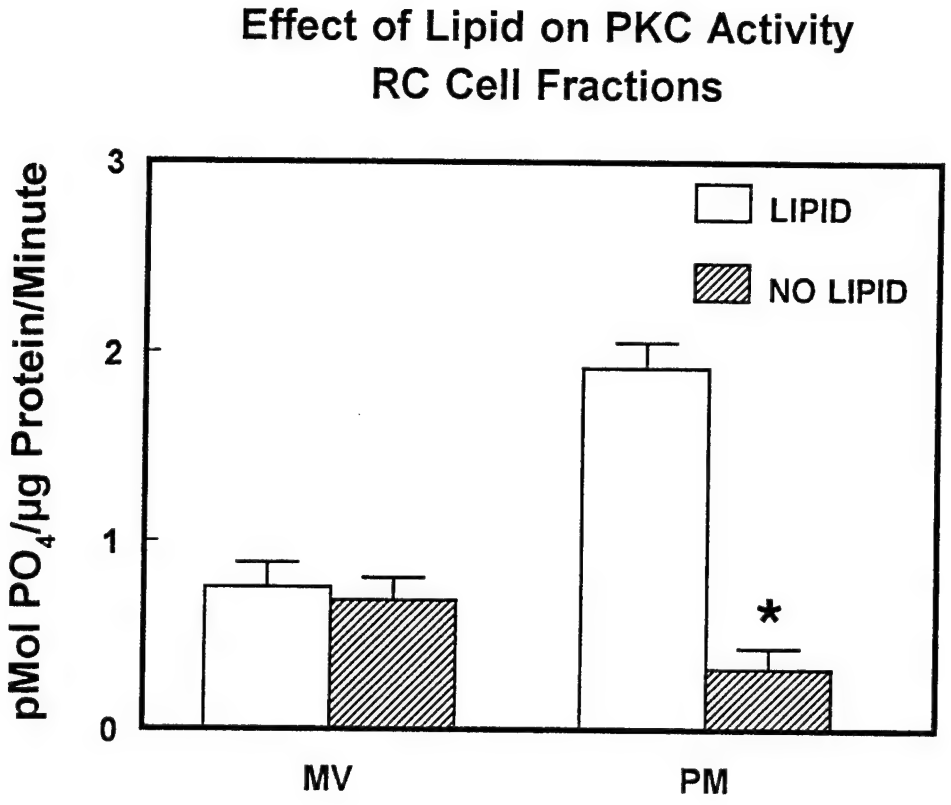

B

Figure 6. EFFECT OF LIPID ON THE PROTEIN KINASE C SPECIFIC ACTIVITY OF MATRIX VESICLES (MV) OR PLASMA MEMBRANES (PM) ISOLATED FROM FOURTH PASSAGE, RESTING ZONE CHONDROCYTE CULTURES. MVs were incubated for 20 minutes at room temperature in RIPA buffer $\pm$ a lipid preparation containing phosphatidylserine and phorbol-12-myristate-13-acetate (PMA) in Triton X-100 mixed micelles prior to the protein kinase C assay. Data represent the mean $\pm$ SEM of PKC activity in membranes from a representative experiment (N=6). *$P < 0.05$, treatment vs. control.


Effect of Lipid on PKC Activity
RC Cell Fractions
pMol PO4/µg Protein/Minute
3
2
1
0
LIPID
NO LIPID
*
MV
PM

1,25-$(OH)_2D_3$, but the effects of the hormone were neither time- nor dose-dependent (Figure 7A). Whereas there was a gradual decrease in control matrix vesicle enzyme activity from 9 to 270 minutes, the effects of 10^{-9} to 10^{-8}M 1,25-$(OH)_2D_3$ were the same at each time examined, reducing PKC activity by nearly 100%. Similarly, 24,25-$(OH)_2D_3$ decreased PKC specific activity of matrix vesicles isolated from resting zone cell cultures in a time- and dose-dependent manner (Figure 8A). At each time point examined, reduction of PKC activity by 10^{-8}M 24,25-$(OH)_2D_3$ was 42.7-54.0%; reduction by 10^{-7}M 24,25-$(OH)_2D_3$ was 68.3-79.7%.

The vitamin D metabolites had the opposite effect on PKC specific activity of isolated plasma membranes. After 9 minutes of exposure to 10^{-9}M 1,25-$(OH)_2D_3$, there was a 532% increase in PKC activity in plasma membranes from growth zone cells (Figure 7B). The effect was maximal since no further increase was observed in plasma membranes exposed to 10^{-8}M 1,25-$(OH)_2D_3$. After 90 minutes, 1,25-$(OH)_2D_3$ was still stimulatory, but the increase was reduced. By 270 minutes, a 66.7% increase was seen only in plasma membranes incubated with 10^{-9}M 1,25-$(OH)_2D_3$. When plasma membranes from resting zone cells were incubated with 24,25-$(OH)_2D_3$, there was a dose- and time-dependent increase in PKC activity (Figure 8B). The 53.3-99.9% increase seen in plasma membranes incubated with 10^{-7}M 24,25-$(OH)_2D_3$ was consistently observed, although the absolute specific activity decreased as a function of time.

Incubation of the membrane fractions with anti-PKCα or anti-PKCζ demonstrated that the vitamin D-sensitive enzyme activity in matrix vesicles isolated from both growth zone and resting zone chondrocyte cultures was PKCζ (Figures 3A, 4A). Anti-PKCζ IgG reduced by 50% the PKC activity in matrix vesicles isolated from growth zone chondrocyte cultures and treated with

Figure 7A. EFFECT OF 1,25-$(OH)_2D_3$ ON PROTEIN KINASE C (PKC) SPECIFIC ACTIVITY OF MATRIX VESICLES ISOLATED FROM CULTURES OF GROWTH ZONE CHONDROCYTES (GC). Membrane fractions were isolated, incubated with control medium or medium containing 10^{-8}M or 10^{-9}M 1,25-$(OH)_2D_3$ for 9, 90, or 270 minutes, and then assayed for PKC specific activity. Data represent the mean $\pm$ SEM of PKC activity in membranes from a representative experiment (N=6). *$P < 0.05$, treatment vs. control.

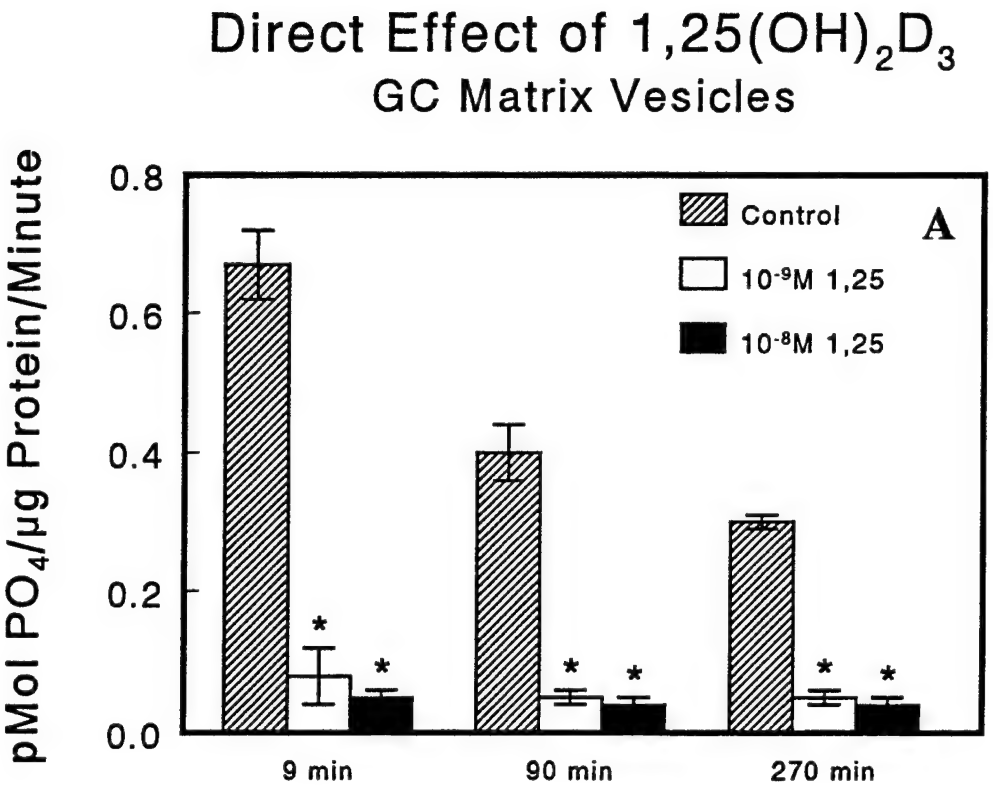

Direct Effect of 1,25(OH)2D3
GC Matrix Vesicles
pMol PO4/µg Protein/Minute
0.8
0.6
0.4
0.2
0.0
Control
10-9M 1,25
10-8M 1,25
A
*
*
*
*
*
*
9 min
90 min
270 min

Figure 7B. EFFECT OF 1,25-$(OH)_2D_3$ ON PROTEIN KINASE C (PKC) SPECIFIC ACTIVITY OF PLASMA MEMBRANES ISOLATED FROM CULTURES OF GROWTH ZONE CHONDROCYTES (GC). Membrane fractions were isolated, incubated with control medium or medium containing 10^{-8}M or 10^{-9}M 1,25-$(OH)_2D_3$ for 9, 90, or 270 minutes, and then assayed for PKC specific activity. Data represent the mean $\pm$ SEM of PKC activity in membranes from a representative experiment (N=6). *$P < 0.05$, treatment vs. control.

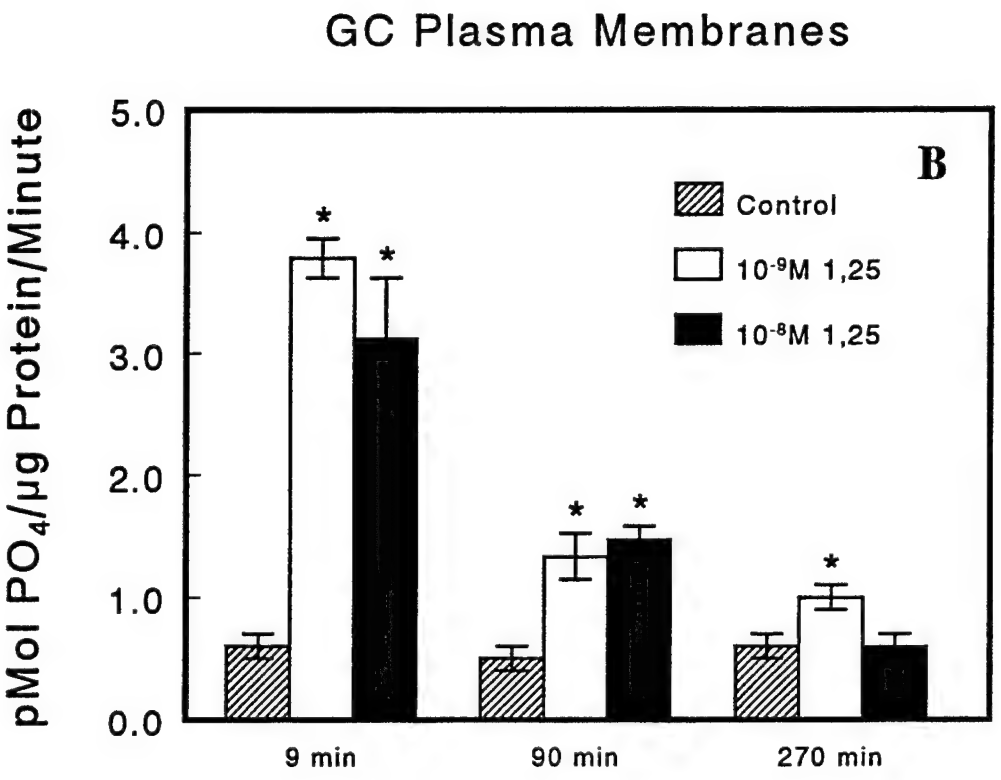

GC Plasma Membranes
pMol PO4/µg Protein/Minute
5.0
4.0
3.0
2.0
1.0
0.0
B
Control
10-9M 1,25
10-8M 1,25
*
9 min
90 min
270 min

Figure 8A. EFFECT OF 24,25-$(OH)_2D_3$ ON PROTEIN KINASE C (PKC) SPECIFIC ACTIVITY OF MATRIX VESICLES ISOLATED FROM CULTURES OF RESTING ZONE CHONDROCYTES (RC). Membrane fractions were isolated, incubated with control medium or medium containing 10^{-8}M or 10^{-7}M 24,25-$(OH)_2D_3$ for 9, 90, or 270 minutes, and then assayed for PKC specific activity. Data represent the mean $\pm$ SEM of PKC activity in membranes from a representative experiment (N=6). *$P<0.05$, treatment vs. control.

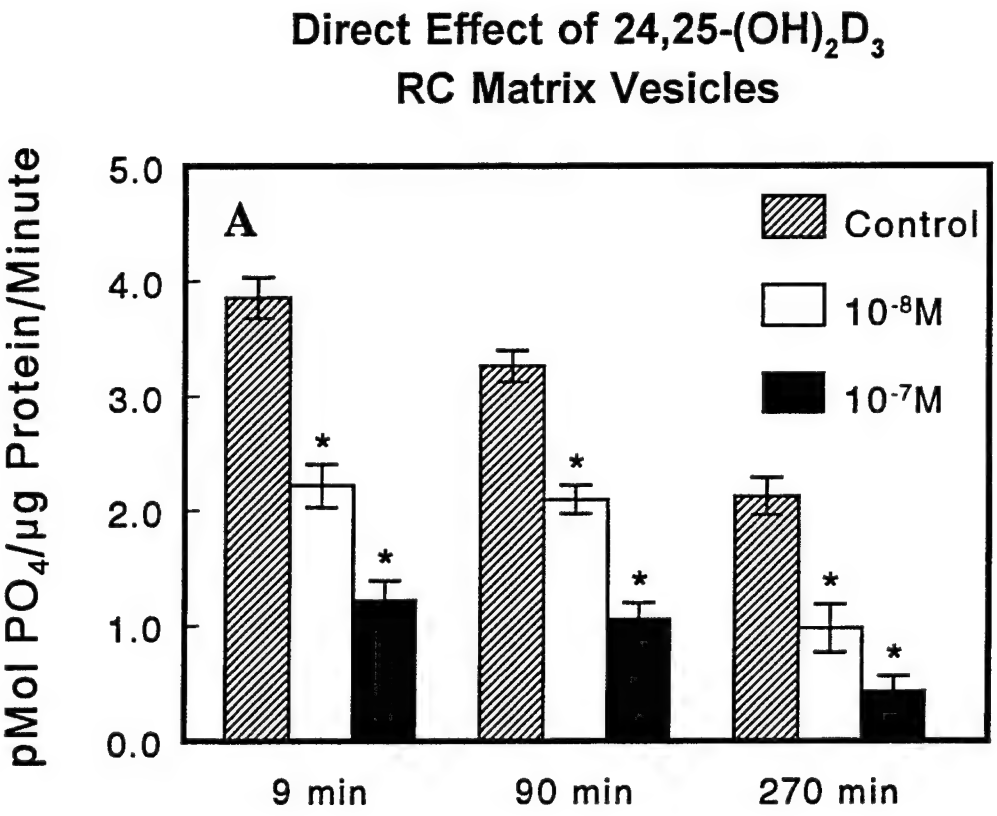
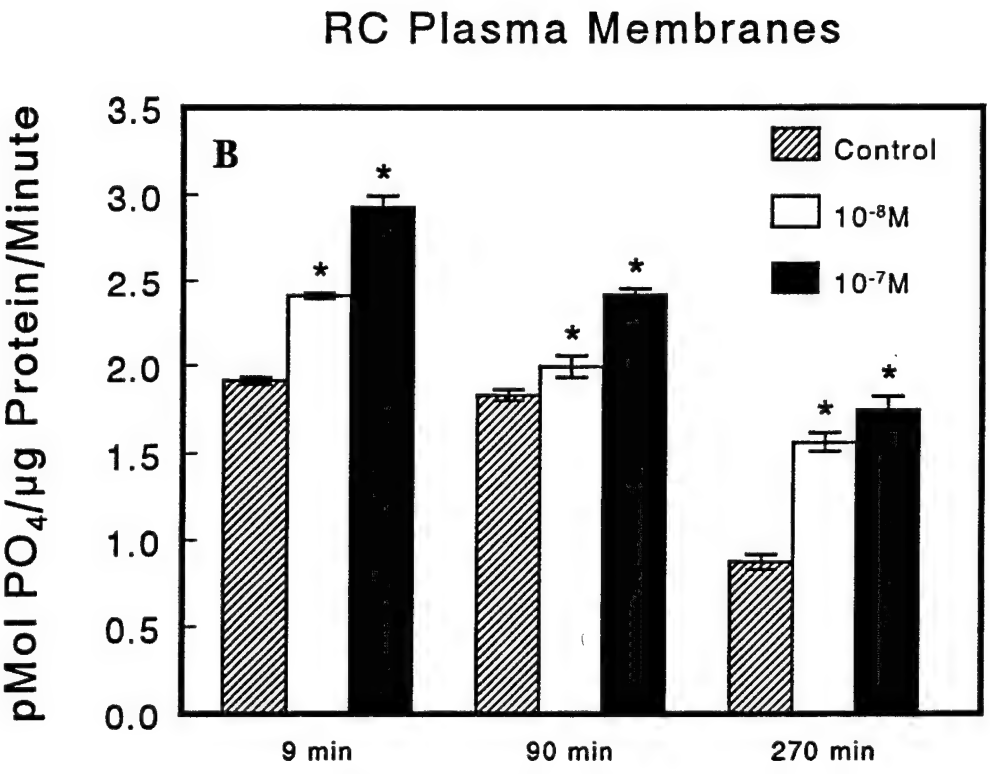

Direct Effect of 24,25-(OH)2D3
RC Matrix Vesicles
pMol PO4/µg Protein/Minute
5.0
4.0
3.0
2.0
1.0
0.0
A
Control
10-8M
10-7M
*
9 min
90 min
270 min

Figure 8B. EFFECT OF 24,25-$(OH)_2D_3$ ON PROTEIN KINASE C (PKC) SPECIFIC ACTIVITY OF PLASMA MEMBRANES ISOLATED FROM CULTURES OF RESTING ZONE CHONDROCYTES (RC). Membrane fractions were isolated, incubated with control medium or medium containing 10^{-8}M or 10^{-7}M 24,25-$(OH)_2D_3$ for 9, 90, or 270 minutes, and then assayed for PKC specific activity. Data represent the mean $\pm$ SEM of PKC activity in membranes from a representative experiment (N=6). *$P<0.05$, treatment vs. control.


RC Plasma Membranes
B
Control
10-8M
10-7M
pMol PO4/µg Protein/Minute
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
9 min
90 min
270 min
*

1,25-$(OH)_2D_3$. Further, enzyme activity in similarly prepared matrix vesicles isolated from resting zone chondrocyte cultures and treated with 24,25-$(OH)_2D_3$ was reduced by 58% after treatment with anti-PKCζ. In contrast, the vitamin D-sensitive enzyme activity in the plasma membranes was PKCα (Figures 3B, 4B). Anti-PKCα IgG decreased PKC activity in growth zone chondrocyte plasma membranes treated with 1,25-$(OH)_2D_3$ by 50% and decreased that of resting zone chondrocyte plasma membranes treated with 24,25-$(OH)_2D_3$ by 77.5%.

The rate of loss of matrix vesicle PKC activity was sensitive to the presence of protease inhibitors in the buffer (Figure 9A, 9B). Inclusion of PMSF as the only protease inhibitor in the RIPA buffer resulted in a 58.8% decline in PKC activity over a 270-minute incubation period. Addition of leupeptin and aprotinin to the RIPA buffer (PIC buffer) decreased the time-dependent loss in PKC activity to 23% over the same time period. PIC buffer enhanced detection of PKC activity in bryostatin-treated matrix vesicles at all time points examined (Figure 9A) compared to samples in RIPA buffer (Figure 9B). The time-dependent loss in matrix vesicle PKC activity was markedly reduced. Moreover, an increased effect of bryostatin on PKC activity was observed.

Figure 9A. EFFECT OF PROTEASE INHIBITOR COCKTAIL ON RESTING ZONE CHONDROCYTE MATRIX VESICLE PROTEIN KINASE C ACTIVITY. Matrix vesicles isolated from confluent, fourth passage resting zone chondrocytes (RC) were incubated with protease inhibitor cocktail (PIC) containing RIPA buffer, aprotinin and leupeptin with or without 10^{-9}-10^{-8}M bryostatin-1 (BRYO) for 9, 90 or 270 minutes prior to assay of protein kinase C. Data represent the mean $\pm$ SEM of PKC activity in matrix vesicles from a representative experiment (N=6). *$P<0.05$, treatment vs. control; $^{\#}P<0.05$, treatment versus 9 minute control.

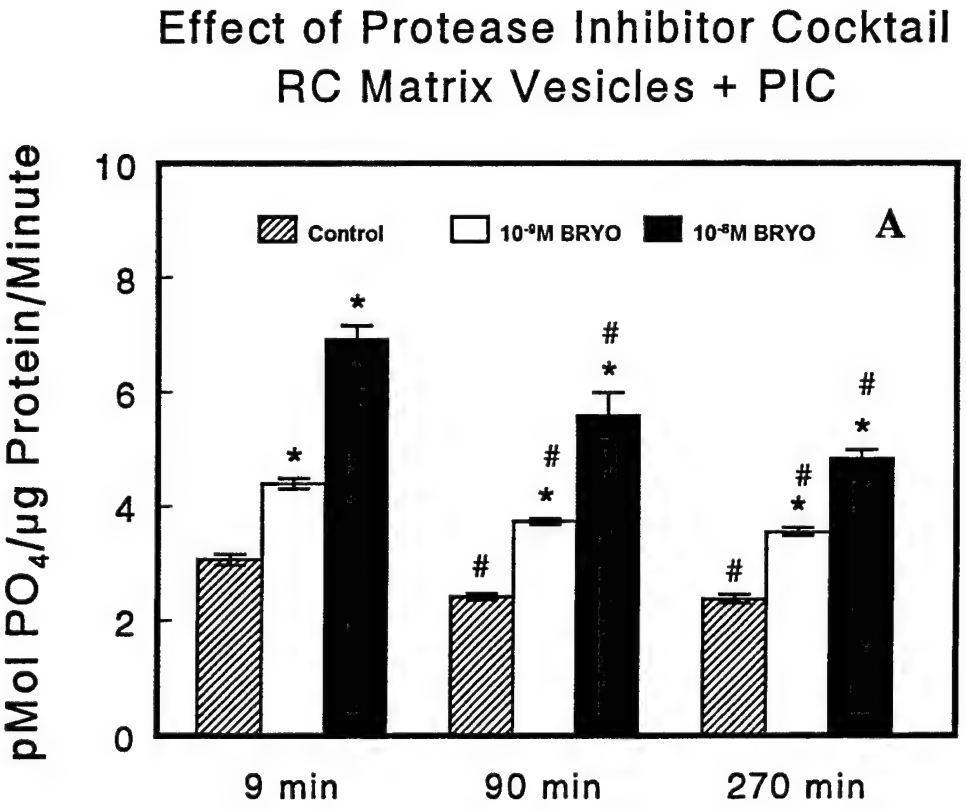
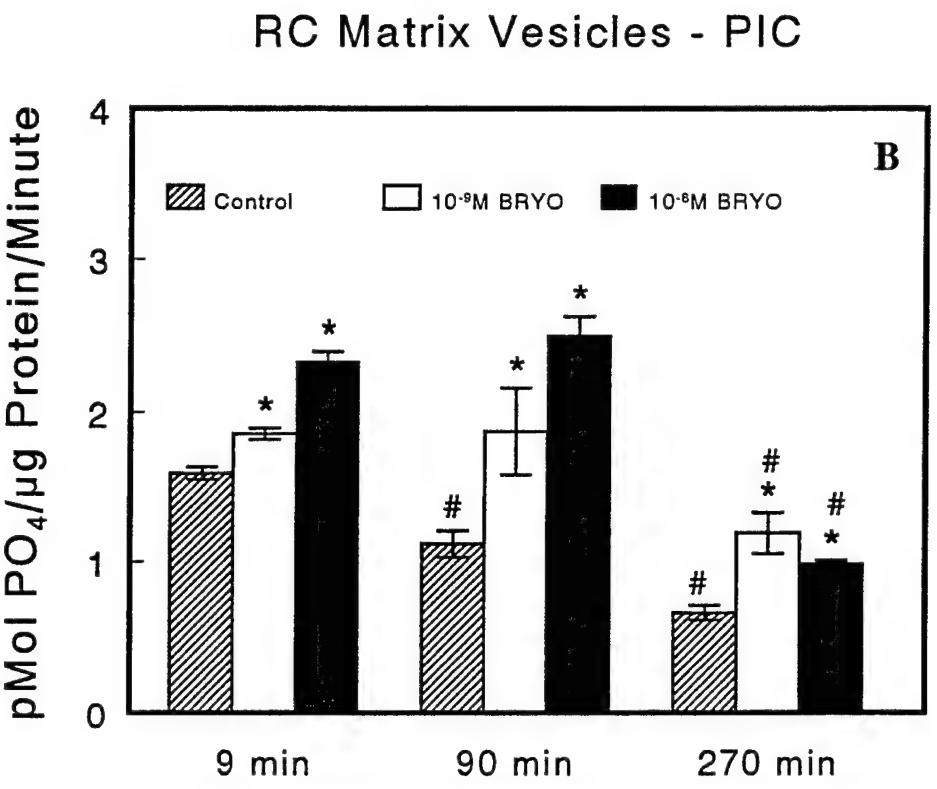

Effect of Protease Inhibitor Cocktail
RC Matrix Vesicles + PIC
pMol PO4/µg Protein/Minute
10
8
6
4
2
0
Control
10-9M BRYO
10-8M BRYO
A
9 min
90 min
270 min

Figure 9B. EFFECT OF PROTEASE INHIBITOR COCKTAIL ON RESTING ZONE CHONDROCYTE MATRIX VESICLE PROTEIN KINASE C ACTIVITY. Matrix vesicles isolated from confluent, fourth passage resting zone chondrocytes (RC) were incubated RIPA buffer alone (-PIC) with or without 10^{-9}-10^{-8}M bryostatin-1 (BRYO) for 9, 90 or 270 minutes prior to assay of protein kinase C. Data represent the mean $\pm$ SEM of PKC activity in matrix vesicles from a representative experiment (N=6). *$P<0.05$, treatment vs. control; $^{\#}P<0.05$, treatment versus 9 minute control.


RC Matrix Vesicles - PIC
B
Control
10-9M BRYO
10-6M BRYO
pMol PO4/µg Protein/Minute
0
1
2
3
4
9 min
90 min
270 min

IV. DISCUSSION AND SUMMARY

Previously, Sylvia and co-workers reported that protein kinase C activity in the cell layer of resting zone and growth zone chondrocytes is regulated by vitamin D metabolites; the effect is metabolite-specific, dose- and time-dependent, and related to the state of chondrogenic maturation *in vivo* (Sylvia *et al.*, 1993) . 1,25-$(OH)_2D_3$ stimulates growth zone chondrocyte protein kinase C activity, while 24,25-$(OH)_2D_3$ stimulates resting zone chondrocyte enzyme activity. The effects in both types of cultures are primarily due to protein kinase Cα. Whereas 1,25-$(OH)_2D_3$ exerts its effect on growth zone chondrocytes via nongenomic mechanisms, stimulation of protein kinase C by 24,25-$(OH)_2D_3$ in resting zone cells involves new gene transcription and protein synthesis (Sylvia *et al.*, 1993).

In the present study, it was shown that matrix vesicles produced by these cells also contain protein kinase C activity. While the plasma membranes of both cell types contain predominantly protein kinase Cα, the matrix vesicles exhibit predominantly protein kinase Cζ. In addition, protein kinase C activity in both membrane fractions from both types of chondrocytes is directly regulated by the target cell-specific vitamin D metabolite. Further, plasma membrane protein kinase C activity is stimulated, while matrix vesicle enzyme activity is inhibited, showing that the nongenomic effect is membrane-dependent. The vitamin D metabolite-sensitive enzyme in plasma membranes is protein kinase Cα; in matrix vesicles, it is protein kinase Cζ.

The finding that matrix vesicles are enriched in the zeta isoform of protein kinase C was unexpected. Matrix vesicles contribute only a minor portion of the total protein kinase C activity detected in cell layer lysates. These extracellular organelles have an average diameter of 20-50nm and are limited to the territorial matrix of the chondrocytes, with a distribution of

approximately 1-2 matrix vesicles/μm^2 of matrix (Schwartz *et al.*, 1991). Any matrix vesicle protein kinase Cζ activity would have been secondary to the dominant plasma membrane protein kinase Cα and, therefore, was not detected in the culture lysates.

The presence of protein kinase Cζ in the matrix vesicles was verified in a number of ways. Anti-PKCζ antibody precipitated the enzyme activity from matrix vesicles.. In contrast, anti-PKCα antibody precipitated PKC activity only from plasma membranes. Furthermore, the protein kinase Cα isoform is known to be activated by calcium and phospholipids, whereas the protein kinase Cζ isoform is calcium-independent and phospholipid-independent (Liyanage *et al.*, 1992; McGlynn *et al.*, 1992). Our observations that matrix vesicle protein kinase C activity was unaltered in the absence of the lipid preparation, while the plasma membrane kinase activity was lipid-dependent, supports the contention that matrix vesicles contain predominantly protein kinase Cζ and plasma membranes contain protein kinase Cα.

Other recent reports (Hug and Sarre, 1993; Liyanage *et al.*, 1992) have demonstrated that the diacylglycerol derived from membrane phospholipids is necessary for function of protein kinase Cα, but not protein kinase Cζ. It is hypothesized that protein kinase Cα translocates to the plasma membrane upon binding to diacylglycerol, while protein kinase Cζ fails to bind diacylglycerol and translocate (Liyanage *et al.*, 1992). The results of the present study suggest that protein kinase Cζ is a constitutive component of matrix vesicles since it is present in those membranes isolated from the extracellular matrix of the chondrocyte cultures, even in the absence of an external stimulus to the cells.

Studies using the protein kinase C activator, bryostatin-1, and the protein kinase pseudosubstrate inhibitor provide further support for the contention that protein kinase C is a constitutive component of matrix vesicles. Bryostatin-1 is known to activate protein kinase C

with the absence of membrane translocation (Grabarek and Ware, 1993), and while protein kinase Cζ lacks a portion of the regulatory domain involved in bryostatin binding (Kazanietz *et al.*, 1994), its activity may still be affected. Similarly, the pseudosubstrate regions of protein kinase Cα and protein kinase Cζ are divergent (Dominguez *et al.*, 1992). Nevertheless, protein kinase Cζ possesses approximately half of the classical pseudosubstrate domain, and therefore may also be inhibited by the classical pseudosubstrate inhibitor.

The presence of protein kinase Cζ was also confirmed by immunoelectron microscopy of isolated matrix vesicles. Matrix vesicles are smaller than the width of the ultrathin section; therefore, only a fraction of the organelles in any field may have been labeled by the immunogold conjugated anti-protein kinase C antibody. It is also possible that protein kinase Cζ was limited to a subset of matrix vesicles. Recent studies suggest that matrix vesicles are heterogenous and may perform a variety of functions in the matrix (Sela *et al.*, 1992). Differential distribution of protein kinase C isoforms may permit the cell to specifically activate or inhibit select subsets via autocrine signaling.

It should be noted that both plasma membranes and matrix vesicles contained both the α and ζ isoforms of protein kinase C. Pretreatment of samples with Protein G-agarose prior to immunoprecipitation produced superior results for visualization of the protein kinase Cα isoform, but impaired detection of the protein kinase Cζ isoform; nonetheless, it is evident that both isoforms were present. Precipitation with anti-protein kinase Cα antibody reduced kinase activity in suspensions of plasma membranes, and precipitation with anti-protein kinase Cζ antibody reduced kinase activity in suspensions of matrix vesicles, suggesting that it is the relative amount of each isoform that is different..

An attractive hypothesis for this quantitative distribution of the α and ζ isoforms is that it

provides a mechanism for differentially transducing autocrine/paracrine hormonal and growth factor signals acting on the cell or on the matrix vesicle. Chondrocytes synthesize and secrete vitamin D metabolites, a process which is regulated by both hormones and growth factors (Yang *et al.*, 1991). Both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ can interact directly with matrix vesicles and plasma membranes, altering fluidity (Swain *et al.*, 1991) and enzyme activity (Schwartz *et al.*,1988b) in a membrane-specific manner. By enriching these membranes, both of which are exposed to the extracellular environment, in different protein kinase C isoforms, the cell has a mechanism for further ensuring that the effects of secreted vitamin D metabolites are appropriate to the target organelle.

In the present study, both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ inhibited protein kinase C in matrix vesicles isolated from their target cell cultures and both metabolites stimulated protein kinase C in their respective target cell plasma membranes. Specificity of response may be provided by the differences in matrix vesicle and plasma membrane composition. Plasma membrane enzymes are differentially distributed between the two membranes (Boyan *et al.*, 1988a; Schwartz *et al.*, 1988b). The phospholipid composition of the two membrane fractions is distinct, and there are cell maturation-specific differences as well (Swain *et al.*, 1992). Phospholipid metabolism in the matrix vesicle is regulated independently of the plasma membrane (Schwartz *et al.*, 1988a). Moreover, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ exert their effects on matrix vesicle and plasma membrane lipids and enzymes in a membrane-dependent, cell maturation-specific manner.

It is clear from the present study that 24,25-$(OH)_2D_3$ has nongenomic, as well as genomic (Sylvia *et al.*, 1993), effects on protein kinase C. The composition of both matrix vesicles and plasma membranes is under genomic control; thus, 24,25-$(OH)_2D_3$ may exert its primary effects

on the resting zone chondrocytes by stimulating new enzyme synthesis and production of new matrix vesicles. Matrix vesicles are regulated independently of the cell, and while their composition may be under genomic control, it is likely that once in the extracellular matrix, they are regulated by direct action of vitamin D metabolites. Detection of 24,25-$(OH)_2D_3$-dependent effects in matrix vesicles has routinely required their isolation and concentration (Boyan *et al.*,1988a; Schwartz *et al.*, 1988b). Similarly, detection of nongenomic effects on the plasma membrane enzymes has required isolation (Schwartz *et al.*, 1988b), presumably due to the ability of the cell to rapidly down-regulate the direct effects.

The results of the present study differ from our previous observations in that the effect of vitamin D on matrix vesicle protein kinase C was opposite that observed in the plasma membrane enzyme. In contrast, when matrix vesicles or plasma membranes from growth zone cell cultures are incubated with 1,25-$(OH)_2D_3$, or when these membrane fractions isolated from resting zone cell cultures are incubated with 24,25-$(OH)_2D_3$, both membranes behave in a comparable manner, at least with respect to alkaline phosphatase specific activity This suggests that the differential distribution of independent isoforms may be key to the distinction in membrane behavior.

The relationship between PKCζ and other vitamin D-dependent matrix vesicle effects is unclear. Protein kinase Cζ may not mediate the effect of 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ on matrix vesicle alkaline phosphatase activity since PKCζ is inhibited, whereas alkaline phosphatase is stimulated. It is also possible that PKCζ phosphorylates an inhibitor of alkaline phosphatase and this is prevented by down regulation of PKC in response to nongenomic regulation by the appropriate vitamin D metabolite.

Our studies also suggest a mechanism for regulating matrix vesicle protein kinase C. Ways

et al. (1992) have shown that protein kinase Cζ is sensitive to proteolysis and that additional leupeptin and aprotonin had to be added to their enzyme buffer in order to detect this isoform. When we added these protease inhibitors to RIPA buffer, not only was matrix vesicle protein kinase C activity elevated, but responses to hormone were enhanced. The matrix vesicles contain active metalloproteinases as well as other proteases (Dean *et al.*, 1992), which may have contributed to the time-dependent loss in kinase activity we observed. These enzymes may play a role in the hormone-dependent inhibition of matrix vesicle protein kinase Cζ activity.

These studies provide the first definitive evidence that vitamin D can exert a nongenomic effect on a signal transduction pathway. Studies using intact cells may demonstrate rapid responses (Civitelli *et al.*, 1990; de Boland and Norman, 1990; Yamaguchi *et al.*, 1987), but the possibility of a genomic mechanism cannot be overruled. Even when inhibitors of gene transcription and protein synthesis are used (Sylvia *et al.*, 1993), the data may not be conclusive. In the present experiments, however, there is no opportunity for misinterpretation since the organelles are isolated from the matrix and contain no DNA or RNA. Moreover, the results suggest an important and novel mechanism for autocrine regulation of extracellular matrix events.

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VITA

Earl Bryan Ellis was born in Midland, Texas on July 17, 1959 to Cecil B. and Nancy Ellis. Dr. Ellis attended Texas A & M University in College Station, Texas and graduated in 1981 magna cum laude with a Bachelor of Science degree in biology. During his undergraduate years, he was elected to membership in the Phi Kappa Phi National Honor Society, and Beta Beta Beta Biology Honor Society. Dr. Ellis received his Doctor of Dental Surgery degree from Baylor College of Dentistry in 1985. During dental school Dr. Ellis received the Psi Omega Scholastic Achievement Award, and was elected to membership in the Omicron Kappa Upsilon National Honor Society. After dental school, Dr. Ellis was commisioned as an officer in the United States Air Force and attended a general practice residency program at Offutt Air Force Base, Nebraska. Following a five year tour of duty in Omaha, Nebraska, Dr. Ellis was reassigned in 1990 to Bitburg Air Base, Germany. In June 1993, Dr. Ellis began graduate training in periodontics at Wilford Hall Medical Center, Lackland Air Force Base, Texas and the University of Texas Health Science Center at San Antonio. Dr. Ellis was married to Barbara C. Squires on 19 December 1992. Dr. Ellis has two daughters, Micah Kathleen Ellis, born 10 October 1983 and Haley Maurine Ellis, born 18 October 1995.